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# Topographical and enzymatic characterization of amylases from the extremely thermophilic eubacterium *Thermotoga maritima*

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The hyperthermophilic eubacterium *Thermotoga maritima* uses starch as a substrate, without releasing amylase activity into the culture medium. The enzyme is associated with the 'toga'. Its expression level is too low to allow the isolation of the pure enzyme. Using cycloheptaamylose and acarbose affinity chromatography and common chromatographic procedures, two enzyme fractions are obtained. They differ in specificity, pH-optimum, temperature dependence and stability. Substrate specificity and $Ca^{2+}$ dependence indicate α-, β- and gluco-amylase activity. Compared with α-amylase from *Bacillus licheniformis* ($T_{max}$ = 75°C), the amylases from *Thermotoga maritima* show exceedingly high thermal stability with an upper temperature limit at 95°C. Significant turnover occurs only between 70 and 100°C, i.e. in the range of viability of the microorganism.

Amylase; *Bacillus licheniformis*; Compartmentation; Thermophilic; *Thermotoga maritima*

## 1. INTRODUCTION

Enzymes isolated from the hyperthermophilic eubacterium *Thermotoga maritima* have been shown to exhibit extreme intrinsic thermal stability [1,2]. For glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the only enzyme which has been purified to homogeneity and characterized in detail, the thermal transition occurs at ≈110°C. Due to the limited data base and the high 'phylogenetic noise' connected with the comparison of homologous sequences, presently, the thermal stability of proteins cannot be correlated with either their amino acid composition or their primary structure [3].

In the present study, attempts were made to isolate and characterize amylases from the hyperthermophilic eubacterium *Thermotoga maritima* with two aims. (i) *Thermotoga maritima* uses starch as a substrate; its catabolism requires amylase activity which – in contrast to other microorganisms such as *Bacillus licheniformis* – is not observed in the growth medium. Thus, the question arises how the macromolecular substrate is taken up by the bacterium; it cannot pass through the channels of the 'toga porins'. (ii) Starch forms a major source of carbohydrate in industrial processes. In technological applications, extremely thermophilic enzymes may be advantageous. Because of its hyperthermophilic nature, and its ability to grow on media containing starch as nutrient, *Thermotoga maritima* is expected to be well-suited to search for thermally stable amylases.

The following questions will be addressed. Where is amylase activity localized, what are the enzymatic characteristics of *Thermotoga* amylases(s), how do *Thermotoga* amylases compare with α-amylase from *Bacillus licheniformis*?

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## 2. MATERIALS AND METHODS

### 2.1. *Chemicals*

DEAE-cellulose (DE 52), Mono-Q, phenyl-Sepharose, butyl-Sepharose and metal-chelating Sepharose were purchased from Whatman (Maidstone) and Pharmacia (Uppsala), respectively. Affinity chromatography made use of cycloheptaamylose (Sigma, St. Louis) and acarbose (Merck, Darmstadt), covalently coupled to epoxy- and aminohexyl-Sepharose (Pharmacia, Uppsala). 2,4-Dinitrosalicylic acid was purchased from Sigma (St. Louis). DNase and lysozyme were products of Boehringer (Mannheim). All other chemicals were analytical-grade substances from Merck (Darmstadt). α-Amylase from *Bacillus licheniformis* was a kind gift of Dr. Mattheijsen, Gent.

### 2.2. *Cultivation of* Thermotoga maritima

Cultivation of *Thermotoga maritima* (MSB8, DSM strain 3109) followed the procedure described by Huber et al. [4]. Cells were harvested in the stationary phase of growth and stored at −70°C.

### 2.3. *Enzyme assay*

Amylase activity was assayed by a modified 2,4-dinitrosalicylic acid (DNSA) method of Bernfeld [5], monitoring the formation of new, free hemiacetal groups at the site of hydrolysis. Enzyme preparations (75 μl) were incubated with a 2% solution of Zulkowsky starch in $H_2O$ (150 μl) and 25 mM triethanolamine buffer pH 5.8 + 2 mM $CaCl_2$ (75 μl) for 20 min at 80°C. The enzyme-reaction mixture was stopped with 1% DNSA in 0.4 N NaOH (300 μl), followed by boiling for 5


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min. Absorbance was determined at 550 nm in an Elisa-reader. A hexokinase assay from Sigma Diagnostics (Darmstadt) was used for specific determination of gluco-amylase and α-glucosidase activity [6].

### 2.4. *Enzyme enrichment*

Frozen cells were thawed in 25 mM triethanolamine buffer pH 5.8 plus 2 mM $CaCl_2$ (standard buffer) and opened using a French press at 136 MPa. DNA was degraded by DNase digestion. Heating the crude extract of the cells for 4 h at 70°C lead to an increase in the volume activity of the amylase(s) by a factor of 1.5–2.0. Whether this effect is caused by the release of enzyme from cell fragments, or whether the enzyme(s) undergo thermal activation is unclear. After removing cell fragments by centrifugation, two-thirds of the total amylase activity was found solube in the supernatant. Activity attached to cell fragments could not be removed by salts or detergents. Soluble amylases were enriched by cycloheptaamylose affinity chromatography in standard buffer, and eluted with cycloheptaamylose. Storing this fraction at 4°C had a purification effect by precipitation of proteins other than amylase. Subsequent acarbose chromatography binds amylases only at about 37°C in the presence of 15% glycerol. An elution with pulses of 0.1 and 1.0 M NaCl separated two different fractions of amylases.

Classical techniques such as ammonium sulfate precipitation, anion exchange chromatography (DEAE, Mono-Q), hydrophobic chromatography (phenyl-Sepharose, butyl-Sepharose), and metal-chelating Sepharose ($Cu^{2+}$-, $Ca^{2+}$-IMAC) did not lead to significant further purification.

### 2.5. *Polyacrylamide gel electrophoresis*

Samples for SDS polyacrylamide gel electrophoresis were prepared by heating in 1% SDS for 20 min. For SDS and native electrophoresis 8–25% gradient gels were used (Phast System, Pharmacia, Uppsala). Gels were stained with silver or Coomassie brilliant blue following procedures recommended by the manufacturer.

Reactivation of amylases in SDS gels was achieved by extensive washing with $H_2O$ and standard buffer [7]. Activity staining of the gels based on the blue iodine-starch reaction [8]. Gels were incubated for 1 h at 40°C and 30 min at 80°C in a solution containing standard buffer + 2% starch. Treating the gels with a solution of 0.5% $I_2$ + 0.5% KI in 50% methanol stopped the enzymatic reaction. Zones of amylase activity appeared as light bands against the dark background of uncleaved starch.

### 2.6. *Preparation of the outer sheaths*

For separating the outer sheaths from cytoplasmic membrane and cytosolar components a Percoll gradient, with a starting concentration of 30%, described by Rachel et al. [9] was used.

### 2.7. *Stability*

pH values of buffers were calibrated at the temperatures used for the respective measurements.

## 3. RESULTS AND DISCUSSION

### 3.1. *Localization of amylase activity*

Spinning down cells of *Thermotoga maritima* in their stationary phase of growth yields the supernatant devoid of measurable amylase activity; taking the total activity after opening the cells in a French Press as 100%, less than 1% amylase activity is found in the growth medium. In contrast, using the same 100% reference, 85% of the total activity is accessible in the surface of the intact (non-lysed) cells, indicating that the major part of amylases must be localized in the 'toga', i.e. the outer sheath of the cell. Sedimentation in a 30% Percoll gradient yields ≥80% of the membrane- and cell-fragment-associated amylase activity in the fractions containing the outer sheath of the cell.

Lysozyme treatment (1 mg/ml, 10 min incubation at 20°C) destroys a thin murein layer stabilizing the protoplast. The cells become coccoid, exhibiting intact cytoplasmic membranes with the 'toga' detached (Fig. 1). Using this approach, no amylase activity is set free, thus excluding the 'periplasmic space' as potential site of amylase action. Since Zulkowsky starch (used as a standard substrate in the assay) has an average molecular mass of 5000 Da, one would assume that the substrate

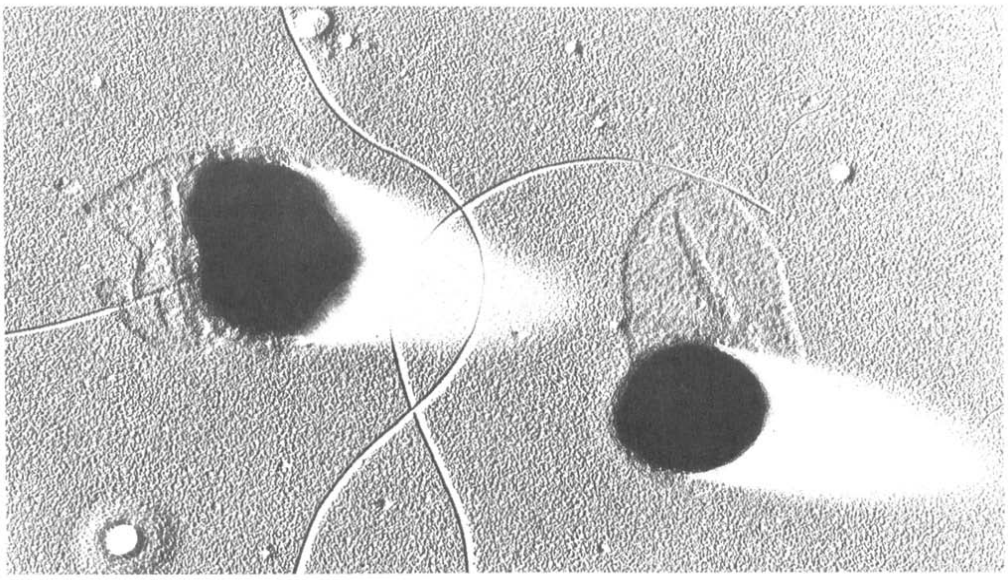

Fig. 1. *Thermotoga maritima* after lysozyme treatment. 1 mg/ml lysozyme, 10 min incubation at 20°C.

Table I

Diagram of *Thermotoga* amylase purification

| | |
|---|---|
| | intact cells (85%) + medium |
| (1) French press<br>(2) DNase-digestion<br>(15 min, 37°C) | |
| | crude preparation (100%) |
| (3) heating step<br>(4 h, 70°C) | |
| | activated crude prep. (170%) |
| (4) centrifugation,<br>washing | |
| | supernatant (67%) pellet (33%) |
| (5) cycloheptaamylose<br>affinity chromatography | |
| | eluted protein (17%) protein not bound (50%) |
| cold precipitation<br>(1–2 h, 4°C) | |
| (6) acarbose chromatography | |
| | eluted protein I(7%) II(7%) protein not bound (19%) |

Percentages refer to relative amylase activity. Preparation buffer: 25 mM triethanolamine pH 5.8 + 2 mM $CaCl_2$ amylase fraction I is eluted with 0.1 M NaCl, fraction II with 1.0 M NaCl.

is unable to pass through the pores of the 'toga' (W. Baumeister and R. Rachel, personal communication). Therefore, we conclude that the major part of the amylase activity must be associated with the surface layer of the bacterium such that the active site of the enzyme is accessible for its substrate, facing the outside of the cell.

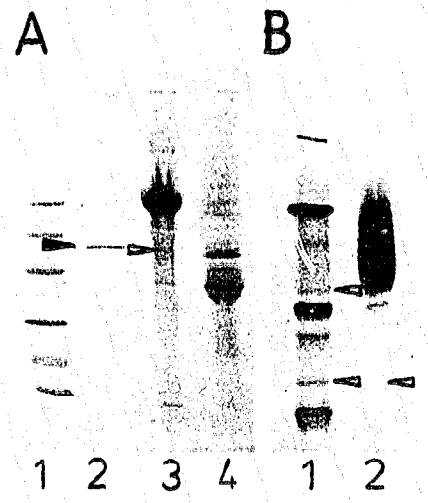


Fig. 2. Electrophoretic characterization of *Thermotoga* amylases. (A) SDS polyacrylamide gel electrophoresis (12% acrylamide). Slot 1: $M_r$ standards (kDa) from top to bottom: phosphorylase *b* (94), bovine serumalbumin (67), ovalbumin (43), carbonic anhydrase (30), trypsin inhibitor (20.1), α-lactalbumin (14.4); slot 2, 3, 4: α-amylase from *B. licheniformis*, and amylase fractions I and II from *Thermotoga maritima*, respectively. (B) Native polyacrylamide gel electrophoresis (8–25% acrylamide). Slots 1,2: fractions I and II from *Thermotoga maritima*. Open and closed arrows point to the location of amylases from *Thermotoga maritima* and *B. licheniformis* as determined by ativity staining.

### 3.2. *Approaches to amylase purification*

The expression level of amylase activity in *Thermotoga maritima* is extremely low. Starting from the maximum yield of cells obtained from 300 liter culture (≈120 g), the total amount of amylases is of the order of 0.5 mg (calculated with the specific activity of *Bacillus licheniformis* α-amylase). Faced with adsorption problems, this is too low to allow the isolation of the enzyme in pure form, especially because the total activity belongs to at least two different enzymes with different

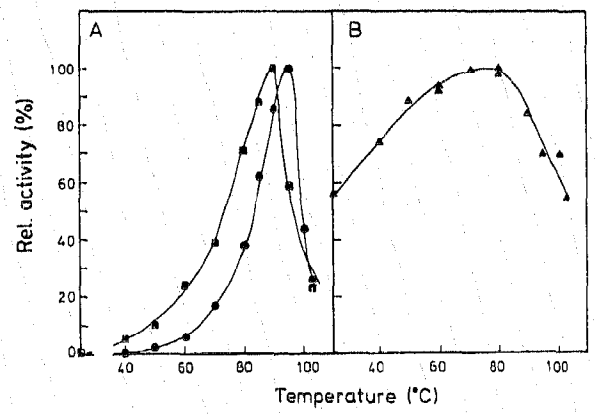


Fig. 3. Amylase activity at different temperature in the enzymatic assay. Incubation: 20 min at optimal pH. (A) *T. maritima* fraction I (●), pH 5.5; *T. maritima* fraction II (■), pH 6.0. (B) *B. licheniformis* α-amylase (▲), pH 6.5.

Table II

Comparison of thermophilic amylases from *Thermotoga maritima* and *Bacillus licheniformis*

| | *Thermotoga maritima* | | *Bacillus licheniformis* |
|---|---|---|---|
| | Fraction I | Fraction II | |
| Optimum temperature (°C) | 95 | 90 | 70–75 |
| Activation energy (kJ/mol) | 98 | 60 | 14 |
| Michaelis constant (%)[a] | 0.23 (80°C) | 0.22 (80°C) | 0.15 (45°C) |
| pH optimum | 5.0 | 6.0 | 6.8 |
| $Ca^{2+}$ dependence | – | + | + |
| Glucoamylase or α-glucosidase activity | – | + | – |
| Inhibition by tendamistat | – | – | – |
| Cross-reaction with AB[b] | – | – | + |

[a] Michaelis constant ($K_m$) in % starch under optimum growth conditions
[b] Cross-reaction with a monoclonal antibody against human salivary α-amylase

substrate specificities (see below). As a consequence, only partial purification of the enzyme(s) could be accomplished. In order to be able to perform extensive studies of the physicochemical properties, cloning of the genes will be required.

Table I gives a schematic diagram of the purification. The following characterization refers to the enzymes eluting from the acarbose column at 0.1 M and 1.0 M NaCl as fraction I and fraction II, respectively. Fig. 2 illustrates the result of SDS and native polyacrylamide gel electrophoresis experiments using Coomassie blue and activity staining to characterize the enzymes. For activity staining, SDS was removed by rinsing the gels with standard buffer (see Materials and Methods). As becomes clear from the position of the bands, fraction I of the *Thermotoga* amylase shows a molecular mass close to the value obtained for the *Bacillus licheniformis* enzyme ($M_r \approx 60$ kDa). Fraction II cannot be reactivated after SDS-polyacrylamide gel electrophoresis. Native gels yield two active bands for fraction I and one for fraction II.

### 3.3. *Characterization and comparison with α-amylase from* Bacillus licheniformis

In contrast to α-amylase from *Bacillus licheniformis*, the amylases from *Thermotoga maritima* exhibit significant catalytic activity only at elevated temperature. As shown in Fig. 3 this is attributable to the exceedingly high activation energies of the *Thermotoga* enzymes as compared to the *Bacillus* enzyme (Table II). Beyond 95°C thermal denaturation occurs, so that reasonable turnover of the *Thermotoga* amylases is only observed between 70 and 100°C, i.e. in the range of viability of the microorganism. Obviously, in the case of *Thermotoga maritima*, one of the reasons for the requirement for high temperature is the fact that the breakdown of nutrients only works at sufficiently high temperatures. The enzyme from *Bacillus licheniformis* shows a much lower activation energy so that activity can be measured even at room temperature, in accordance with the mesophilic or moderately thermophilic nature of the organism.

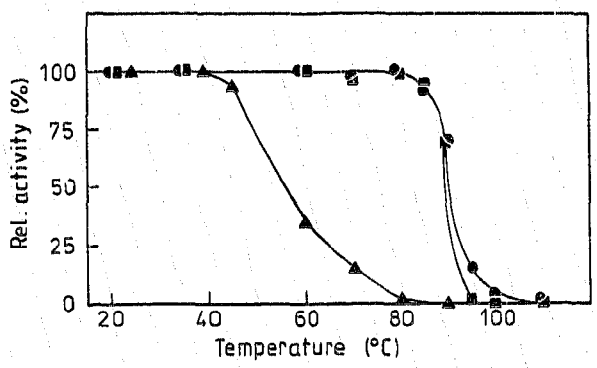


Fig. 4. Transitions of thermal inactivation. Incubation time: 30 min; standard test: 20 min at 45°C and 80°C, respectively. Symbols as in Fig. 3.

As shown by heat inactivation experiments where the residual activity at the respective temperatures was measured in the absence and in the presence of trypsin [10,11], thermal inactivation of the *Thermotoga* amylases is irreversible. Thus, thermodynamic stability parameters from equilibrium transitions are inaccessible. In order to give a semi-quantitative measure of the thermal stability of the enzymes, final values of residual activity after sufficiently long incubation were applied to characterize the 'transition midpoints' of heat denaturation. The $T_M$-values for α-amylase from *Bacillus licheniformis* and for the *Thermotoga* enzymes are 55°C and ≈90°C, respectively (Fig. 4).

The pH profiles for enzyme catalyses are given in Fig. 5; compared to the *Bacillus* amylase, the *Thermotoga* amylase exhibit a slight shift toward the acidic pH range. This cannot be correlated with differences in specificity, since fractions I and II obviously differ in this respect. As taken from the $Ca^{2+}$ dependence and the hydrolysis of specific substrates, fraction I is a β-amylase, while fraction II shows both α-amylase and glucoamylase activity (Table II). Attempts to confirm the findings of the catalytic experiments by specific binding studies using inhibitors and antibodies failed: tendamistat, a potent α-amylase inhibitor, does not bind to the *Thermotoga*

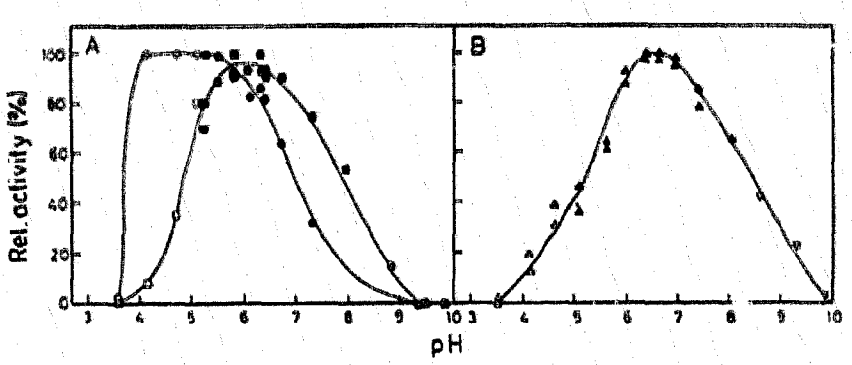


Fig. 5. pH-dependence of amylase activity measured at varying pH in the 20 min standard test. (△, ○, □) 100 mM citrate-HCl + 1 mM $CaCl_2$; (▲, ●, ■) 100 mM Tris-maleate + 1 mM $CaCl_2$; (▽, ⌀, ⌀) 100 mM glycine-NaOH + $CaCl_2$. (A) *T. maritima* fraction I (○, ●, ⌀), 80°C; *T. maritima* fraction II (□, ■, ⌀), 80°C. (B) *B. licheniformis* α-amylase (△, ▲, ▽), 45°C.

*maritima* enzymes; the same holds for an α-amylase antibody produced against human salivary α-amylase. Whether this is caused by structural differences of the enzymes, or whether fraction II consists mainly of glucoamylase, thus hiding α-specific effects, remains open.

## 4. CONCLUSIONS

*Thermotoga maritima* contains amylases of at least two different specificities. None of them is secreted into the growth medium; instead, evidence from fractionation experiments and lysozyme digestion shows that the enzymes are attached to the 'toga'. This location seems plausible faced with the dimensions of the pores in the surface layer: starch as a nutrient must be degraded in order to pass the outer sheats, i.e. to enter the compartment of its metabolic use. Comparison of the physical properties of the amylases from *Bacillus licheniformis* and *Thermotoga maritima* proves the hyperthermophilic nature of the *Thermotoga* enzymes, which for this reason seem to be ideal candidates for industrial starch processing. Unfortunately the expression of the enzymes under optimum growth conditions is too low to produce sufficient amounts of the enzymes. Therefore, cloning the genes seems the only way to proceed toward technical application.

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